

TITLE
METHOD FOR THE PRODUCTION OF GLYCEROL
BY RECOMBINANT ORGANISMS

FIELD OF INVENTION

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AI The present invention relates to the field of molecular biology and the use of recombinant organisms for the production of glycerol and compounds derived from the glycerol biosynthetic pathway. More specifically the invention describes the construction of a recombinant cell for the production of glycerol and derived compounds from a carbon substrate, the cell containing foreign
10 genes encoding proteins having glycerol-3-phosphate dehydrogenase (G3PDH) and glycerol-3-phosphatase (G3P phosphatase) activities where the endogenous genes encoding the glycerol-converting glycerol kinase and glycerol dehydrogenase activities have been deleted.

BACKGROUND

15 Glycerol is a compound in great demand by industry for use in cosmetics, liquid soaps, food, pharmaceuticals, lubricants, anti-freeze solutions, and in numerous other applications. The esters of glycerol are important in the fat and oil industry. Historically, glycerol has been isolated from animal fat and similar sources; however, the process is laborious and inefficient. Microbial
20 production of glycerol is preferred.

Not all organisms have a natural capacity to synthesize glycerol. However, the biological production of glycerol is known for some species of bacteria, algae, and yeast. The bacteria *Bacillus licheniformis* and *Lactobacillus*
25 *lycopersica* synthesize glycerol. Glycerol production is found in the halotolerant algae *Dunaliella* sp. and *Asteromonas gracilis* for protection against high external salt concentrations (Ben-Amotz et al., (1982) *Experientia* 38:49-52). Similarly, various osmotolerant yeast synthesize glycerol as a protective measure. Most strains of *Saccharomyces* produce some glycerol during alcoholic fermentation and this production can be increased by the application of
30 osmotic stress (Albertyn et al., (1994) *Mol. Cell. Biol.* 14, 4135-4144). Earlier this century glycerol was produced commercially with *Saccharomyces* cultures to which steering reagents were added such as sulfites or alkalis. Through the formation of an inactive complex, the steering agents block or inhibit the conversion of acetaldehyde to ethanol; thus, excess reducing equivalents
35 (NADH) are available to or "steered" towards dihydroxyacetone phosphate (DHAP) for reduction to produce glycerol. This method is limited by the partial inhibition of yeast growth that is due to the sulfites. This limitation can be partially overcome by the use of alkalis which create excess NADH equivalents by a different mechanism. In this practice, the alkalis initiated a Cannizarro

disproportionation to yield ethanol and acetic acid from two equivalents of acetaldehyde. Thus, although production of glycerol is possible from naturally occurring organisms, production is often subject to the need to control osmotic stress of the cultures and the production of sulfites. A method free from these limitations is desirable. Production of glycerol from recombinant organisms containing foreign genes encoding key steps in the glycerol biosynthetic pathway is one possible route to such a method.

A number of the genes involved in the glycerol biosynthetic pathway have been isolated. For example, the gene encoding glycerol-3-phosphate dehydrogenase (DAR1, GPD1) has been cloned and sequenced from *Saccharomyces diastaticus* (Wang et al., (1994), *J. Bact.* 176:7091-7095). The DAR1 gene was cloned into a shuttle vector and used to transform *E. coli* where expression produced active enzyme. Wang et al., *supra*, recognizes that DAR1 is regulated by the cellular osmotic environment but does not suggest how the gene might be used to enhance glycerol production in a recombinant organism.

Other glycerol-3-phosphate dehydrogenase enzymes have been isolated. For example, sn-glycerol-3-phosphate dehydrogenase has been cloned and sequenced from *S. cerevisiae* (Larason et al., (1993) *Mol. Microbiol.*, 10:1101). Albertyn et al., (1994) *Mol. Cell. Biol.*, 14:4135) teach the cloning of GPD1 encoding a glycerol-3-phosphate dehydrogenase from *S. cerevisiae*. Like Wang et al., both Albertyn et al. and Larason et al. recognize the osmo-sensitivity of the regulation of this gene but do not suggest how the gene might be used in the production of glycerol in a recombinant organism.

As with G3PDH, glycerol-3-phosphatase has been isolated from *Saccharomyces cerevisiae* and the protein identified as being encoded by the GPP1 and GPP2 genes (Norbeck et al., (1996) *J. Biol. Chem.*, 271:13875). Like the genes encoding G3PDH, it appears that GPP2 is osmotically-induced.

Although the genes encoding G3PDH and G3P phosphatase have been isolated, there is no teaching in the art that demonstrates glycerol production from recombinant organisms with G3PDH/G3P phosphatase expressed together or separately. Further, there is no teaching to suggest that efficient glycerol production from any wild-type organism is possible using these two enzyme activities that does not require applying some stress (salt or an osmolyte) to the cell. In fact, the art suggests that G3PDH activities may not affect glycerol production. For example, Eustace ((1987), *Can. J. Microbiol.*, 33:112-117)) teaches hybridized yeast strains that produced glycerol at greater levels than the parent strains. However, Eustace also demonstrates that G3PDH activity remained constant or slightly lower in the hybridized strains as opposed to the wild type.

Glycerol is an industrially useful material. However, other compounds may be derived from the glycerol biosynthetic pathway that also have commercial significance. For example, glycerol-producing organisms may be engineered to produce 1,3-propanediol (U.S. 5686276), a monomer having potential utility in the production of polyester fibers and the manufacture of polyurethanes and cyclic compounds. It is known for example that in some organisms, glycerol is converted to 3-hydroxypropionaldehyde and then to 1,3-propanediol through the actions of a dehydratase enzyme and an oxidoreductase enzyme, respectively. Bacterial strains able to produce 1,3-propanediol have been found, for example, in the groups *Citrobacter*, *Clostridium*, *Enterobacter*, *Ilyobacter*, *Klebsiella*, *Lactobacillus*, and *Pelobacter*. Glycerol dehydratase and diol dehydratase systems are described by Seyfried et al. (1996) *J. Bacteriol.* 178:5793-5796 and Tobimatsu et al. (1995) *J. Biol. Chem.* 270:7142-7148, respectively. Recombinant organisms, containing exogenous dehydratase enzyme, that are able to produce 1,3-propanediol have been described (U.S. 5686276). Although these organisms produce 1,3-propanediol, it is clear that they would benefit from a system that would minimize glycerol conversion.

There are a number of advantages in engineering a glycerol-producing organism for the production of 1,3-propanediol where conversion of glycerol is minimized. A microorganism capable of efficiently producing glycerol under physiological conditions is industrially desirable, especially when the glycerol itself will be used as a substrate *in vivo* as part of a more complex catabolic or biosynthetic pathway that could be perturbed by osmotic stress or the addition of steering agents (e.g., the production of 1,3-propanediol). Some attempts at creating glycerol kinase and glycerol dehydrogenase mutants have been made. For example, De Koning et al. (1990) *Appl. Microbiol Biotechnol.* 32:693-698 report the methanol-dependent production of dihydroxyacetone and glycerol by mutants of the methylotrophic yeast *Hansenula polymorpha* blocked in dihydroxyacetone kinase and glycerol kinase. Methanol and an additional substrate, required to replenish the xyulose-5-phosphate co-substrate of the assimilation reaction, were used to produce glycerol; however, a dihydroxyacetone reductase (glycerol dehydrogenase) is also required. Similarly, Shaw and Cameron, Book of Abstracts, 211th ACS National Meeting, New Orleans, LA, March 24-28 (1996), BIOT-154 Publisher: American Chemical Society, Washington, D. C., investigate the deletion of *ldhA* (lactate dehydrogenase), *glpK* (glycerol kinase), and *tpiA* (triosephosphate isomerase) for the optimization of 1,3-propanediol production. They do not suggest the expression of cloned genes for G3PDH or G3P phosphatase for the

production of glycerol or 1,3-propanediol and they do not discuss the impact of glycerol dehydrogenase.

The problem to be solved, therefore, is the lack of a process to direct carbon flux towards glycerol production by the addition or enhancement of certain enzyme activities, especially G3PDH and G3P phosphatase which respectively catalyze the conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P) and then to glycerol. The problem is complicated by the need to control the carbon flux away from glycerol by deletion or decrease of certain enzyme activities, especially glycerol kinase and glycerol dehydrogenase which respectively catalyze the conversion of glycerol plus ATP to G3P and glycerol to dihydroxyacetone (or glyceraldehyde).

SUMMARY OF THE INVENTION

The present invention provides a method for the production of glycerol from a recombinant organism comprising: transforming a suitable host cell with an expression cassette comprising either one or both of (a) a gene encoding a protein having glycerol-3-phosphate dehydrogenase activity and (b) a gene encoding a protein having glycerol-3-phosphate phosphatase activity, where the suitable host cell contains a disruption in either one or both of (a) a gene encoding an endogenous glycerol kinase and (b) a gene encoding an endogenous glycerol dehydrogenase, wherein the disruption prevents the expression of active gene product; culturing the transformed host cell in the presence of at least one carbon source selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and single-carbon substrates, whereby glycerol is produced; and recovering the glycerol produced.

The present invention further provides a process for the production of 1,3-propanediol from a recombinant organism comprising: transforming a suitable host cell with an expression cassette comprising either one or both of (a) a gene encoding a protein having glycerol-3-phosphate dehydrogenase activity and (b) a gene encoding a protein having glycerol-3-phosphate phosphatase activity, the suitable host cell having at least one gene encoding a protein having a dehydratase activity and having a disruption in either one or both of (a) a gene encoding an endogenous glycerol kinase and (b) a gene encoding an endogenous glycerol dehydrogenase, wherein the disruption in the genes of (a) or (b) prevents the expression of active gene product; culturing the transformed host cell in the presence of at least one carbon source selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and single-carbon substrates whereby 1,3-propanediol is produced; and recovering the 1,3-propanediol produced.

Additionally, the invention provides for a process for the production of 1,3-propanediol from a recombinant organism where multiple copies of endogeneous genes are introduced.

Further embodiments of the invention include host cells transformed with heterologous genes for the glycerol pathway as well as host cells which contain endogeneous genes for the glycerol pathway.

Additionally, the invention provides recombinant cells suitable for the production either glycerol or 1,3-propanediol, the host cells having genes expressing either one or both of a glycerol-3-phosphate dehydrogenase activity and a glycerol-3-phosphate phosphatase activity wherein the cell also has disruptions in either one or both of a gene encoding an endogenous glycerol kinase and a gene encoding an endogenous glycerol dehydrogenase, wherein the disruption in the genes prevents the expression of active gene product.

BRIEF DESCRIPTION OF THE FIGURES, BIOLOGICAL DEPOSITS AND SEQUENCE LISTING

Figure 1 illustrates the representative enzymatic pathways involving glycerol metabolism.

Applicants have made the following biological deposits under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure:

Depositor Identification Reference	Int'l. Depository Designation	Date of Deposit
<i>Escherichia coli</i> pAH21/DH5 α (containing the GPP2 gene)	ATCC 98187	26 September 1996
<i>Escherichia coli</i> (pDAR1A/AA200) (containing the DAR1 gene)	ATCC 98248	6 November 1996
FM5 <i>Escherichia coli</i> RJF10m (containing a <i>glpK</i> disruption)	ATCC 98597	25 November 1997
FM5 <i>Escherichia coli</i> MSP33.6 (containing a <i>gldA</i> disruption)	ATCC 98598	25 November 1997

"ATCC" refers to the American Type Culture Collection international depository located at 10801 University Blvd., Manassas, VA 20108-2209, 12301 Parklawn Drive, Rockville, MD 20852 U.S.A. The designation is the accession number of the deposited material.

Applicants have provided 43 sequences in conformity with the Rules for the Standard Representation of Nucleotide and Amino Acid Sequences in Patent Applications (Annexes I and II to the Decision of the President of the EPO, published in Supplement No. 2 to OJ EPO, 12/1992) and with 37 C.F.R. 1.821-1.825 and Appendices A and B (Requirements for Application Disclosures Containing Nucleotides and/or Amino Acid Sequences).

DETAILED DESCRIPTION OF THE INVENTION

The present invention solves the problem stated above by providing a method for the biological production of glycerol from a fermentable carbon source in a recombinant organism. The method provides a rapid, inexpensive and environmentally-responsible source of glycerol useful in the cosmetics and pharmaceutical industries. The method uses a microorganism containing cloned homologous or heterologous genes encoding glycerol-3-phosphate dehydrogenase (G3PDH) and/or glycerol-3-phosphatase (G3P phosphatase). These genes are expressed in a recombinant host having disruptions in genes encoding endogenous glycerol kinase and/or glycerol dehydrogenase enzymes. The method is useful for the production of glycerol, as well as any end products for which glycerol is an intermediate. The recombinant microorganism is contacted with a carbon source and cultured and then glycerol or any end products derived therefrom are isolated from the conditioned media. The genes may be incorporated into the host microorganism separately or together for the production of glycerol.

Applicants' process has not previously been described for a recombinant organism and required the isolation of genes encoding the two enzymes and their subsequent expression in a host cell having disruptions in the endogenous kinase and dehydrogenase genes. It will be appreciated by those familiar with this art that Applicants' process may be generally applied to the production compounds where glycerol is a key intermediate, e.g., 1,3-propanediol.

As used herein the following terms may be used for interpretation of the claims and specification.

The terms "glycerol-3-phosphate dehydrogenase" and "G3PDH" refer to a polypeptide responsible for an enzyme activity that catalyzes the conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P). *In vivo* G3PDH may be NADH; NADPH; or FAD-dependent. The NADH-dependent enzyme (EC 1.1.1.8) is encoded, for example, by several genes including GPD1 (GenBank Z74071x2), or GPD2 (GenBank Z35169x1), or GPD3 (GenBank G984182), or DAR1 (GenBank Z74071x2). The NADPH-dependent enzyme (EC 1.1.1.94) is encoded by *gpsA* (GenBank U321643, (cds 197911-196892) G466746 and L45246). The FAD-dependent enzyme (EC 1.1.99.5) is encoded by GUT2 (GenBank Z47047x23), or glpD (GenBank G147838), or glpABC (GenBank M20938).

The terms "glycerol-3-phosphatase", "sn-glycerol-3-phosphatase", or "d,l-glycerol phosphatase", and "G3P phosphatase" refer to a polypeptide responsible for an enzyme activity that catalyzes the conversion of glycerol-3-phosphate and water to glycerol and inorganic phosphate. G3P phosphatase is

encoded, for example, by GPP1 (GenBank Z47047x125), or GPP2 (GenBank U18813x11).

5 The term "glycerol kinase" refers to a polypeptide responsible for an enzyme activity that catalyzes the conversion of glycerol and ATP to glycerol-3-phosphate and ADP. The high energy phosphate donor ATP may be replaced by physiological substitutes (e.g. phosphoenolpyruvate). Glycerol kinase is encoded, for example, by GUT1 (GenBank U11583x19) and *glpK* (GenBank L19201).

10 The term "glycerol dehydrogenase" refers to a polypeptide responsible for an enzyme activity that catalyzes the conversion of glycerol to dihydroxyacetone (E.C. 1.1.1.6) or glycerol to glyceraldehyde (E.C. 1.1.1.72). A polypeptide responsible for an enzyme activity that catalyzes the conversion of glycerol to dihydroxyacetone is also referred to as a "dihydroxyacetone reductase". Glycerol dehydrogenase may be dependent upon NADH
15 (E.C. 1.1.1.6), NADPH (E.C. 1.1.1.72), or other cofactors (e.g., E.C. 1.1.1.99.22). A NADH-dependent glycerol dehydrogenase is encoded, for example, by *gldA* (GenBank U00006).

The term "dehydratase enzyme" will refer to any enzyme that is capable of isomerizing or converting a glycerol molecule to the product
20 3-hydroxypropion-aldehyde. For the purposes of the present invention the dehydratase enzymes include a glycerol dehydratase (E.C. 4.2.1.30) and a diol dehydratase (E.C. 4.2.1.28) having preferred substrates of glycerol and 1,2-propanediol, respectively. In *Citrobacter freundii*, for example, glycerol dehydratase is encoded by three polypeptides whose gene sequences are
25 represented by *dhaB*, *dhaC* and *dhaE* (GenBank U09771: base pairs 8556-10223, 10235-10819, and 10822-11250, respectively). In *Klebsiella oxytoca*, for example, diol dehydratase is encoded by three polypeptides whose gene sequences are represented by *pddA*, *pddB*, and *pddC* (GenBank D45071: base pairs 121-1785, 1796-2470, and 2485-3006, respectively).

30 The terms "GPD1", "DAR1", "OSG1", "D2830", and "YDL022W" will be used interchangeably and refer to a gene that encodes a cytosolic glycerol-3-phosphate dehydrogenase and is characterized by the base sequence given as SEQ ID NO:1.

35 The term "GPD2" refers to a gene that encodes a cytosolic glycerol-3-phosphate dehydrogenase and is characterized by the base sequence given in SEQ ID NO:2.

The terms "GUT2" and "YIL155C" are used interchangeably and refer to a gene that encodes a mitochondrial glycerol-3-phosphate dehydrogenase and is characterized by the base sequence given in SEQ ID NO:3.

The terms "GPP1", "RHR2" and "YIL053W" are used interchangeably and refer to a gene that encodes a cytosolic glycerol-3-phosphatase and is characterized by the base sequence given in SEQ ID NO:4.

5 The terms "GPP2", "HOR2" and "YER062C" are used interchangeably and refer to a gene that encodes a cytosolic glycerol-3-phosphatase and is characterized by the base sequence given as SEQ ID NO:5.

10 The term "GUT1" refers to a gene that encodes a cytosolic glycerol kinase and is characterized by the base sequence given as SEQ ID NO:6. The term "*glpK*" refers to another gene that encodes a glycerol kinase and is characterized by the base sequence given in GeneBank L19201, base pairs 77347-78855.

15 The term "*gldA*" refers to a gene that encodes a glycerol dehydrogenase and is characterized by the base sequence given in GeneBank U00006, base pairs 3174-4316. The term "*dhaD*" refers to another gene that encodes a glycerol dehydrogenase and is characterized by the base sequence given in GeneBank U09771, base pairs 2557-3654.

20 As used herein, the terms "function" and "enzyme function" refer to the catalytic activity of an enzyme in altering the energy required to perform a specific chemical reaction. Such an activity may apply to a reaction in equilibrium where the production of both product and substrate may be accomplished under suitable conditions.

The terms "polypeptide" and "protein" are used interchangeably.

25 The terms "carbon substrate" and "carbon source" refer to a carbon source capable of being metabolized by host organisms of the present invention and particularly mean carbon sources selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and one-carbon substrates or mixtures thereof.

30 "Conversion" refers to the metabolic processes of an organism or cell that by means of a chemical reaction degrades or alters the complexity of a chemical compound or substrate.

The terms "host cell" and "host organism" refer to a microorganism capable of receiving foreign or heterologous genes and additional copies of endogeneous genes and expressing those genes to produce an active gene product.

35 The terms "production cell" and "production organism" refer to a cell engineered for the production of glycerol or compounds that may be derived from the glycerol biosynthetic pathway. The production cell will be recombinant and contain either one or both of a gene that encodes a protein having a glycerol-3-phosphate dehydrogenase activity and a gene encoding a

protein having a glycerol-3-phosphatase activity. In addition to the G3PDH and G3P phosphatase genes, the host cell will contain dislocations in one or both of a gene encoding an endogenous glycerol kinase and a gene encoding an endogenous glycerol dehydrogenase. Where the production cell is designed to produce 1,3-propanediol, it will additionally contain a gene encoding a protein having a dehydratase activity.

The terms "foreign gene", "foreign DNA", "heterologous gene", and "heterologous DNA" all refer to genetic material native to one organism that has been placed within a different host organism.

The term "endogenous" as used herein with reference to genes or polypeptides expressed by genes, refers to genes or polypeptides that are native to a production cell and are not derived from another organism. Thus an "endogenous glycerol kinase" and an "endogenous glycerol dehydrogenase" are terms referring to polypeptides encoded by genes native to the production cell.

The terms "recombinant organism" and "transformed host" refer to any organism transformed with heterologous or foreign genes. The recombinant organisms of the present invention express foreign genes encoding G3PDH and G3P phosphatase for the production of glycerol from suitable carbon substrates. Additionally, the terms "recombinant organism" and "transformed host" refer to any organism transformed with endogenous (or homologous) genes so as to increase the copy number of the genes.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding) and following (3' non-coding) the coding region. The terms "native" and "wild-type" gene refer to the gene as found in nature with its own regulatory sequences.

The terms "encoding" and "coding" refer to the process by which a gene, through the mechanisms of transcription and translation, produces an amino acid sequence. The process of encoding a specific amino acid sequence is meant to include DNA sequences that may involve base changes that do not cause a change in the encoded amino acid, or which involve base changes which may alter one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. Therefore, the invention encompasses more than the specific exemplary sequences. Modifications to the sequence, such as deletions, insertions, or substitutions in the sequence which produce silent changes that do not substantially affect the functional properties of the resulting protein molecule are also contemplated. For example, alterations in the gene sequence which reflect the degeneracy of the genetic code, or which result in the production of a chemically equivalent amino acid at a given site, are contemplated; thus, a codon for the amino acid alanine, a

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hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a biologically equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. In some cases, it may in fact be desirable to make mutants of the sequence in order to study the effect of alteration on the biological activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity in the encoded products. Moreover, the skilled artisan recognizes that sequences encompassed by this invention are also defined by their ability to hybridize, under stringent conditions (0.1X SSC, 0.1% SDS, 65 °C), with the sequences exemplified herein.

The term "expression" refers to the transcription and translation to gene product from a gene coding for the sequence of the gene product.

The terms "plasmid", "vector", and "cassette" as used herein refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Transformation cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitate transformation of a particular host cell. "Expression cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

The terms "transformation" and "transfection" refer to the acquisition of new genes in a cell after the incorporation of nucleic acid. The acquired genes may be integrated into chromosomal DNA or introduced as extrachromosomal replicating sequences. The term "transformant" refers to the cell resulting from a transformation.

The term "genetically altered" refers to the process of changing hereditary material by transformation or mutation. The terms "disruption" and "gene interrupt" as applied to genes refer to a method of genetically altering an organism by adding to or deleting from a gene a significant portion of that gene such that the protein encoded by that gene is either not expressed or not expressed in active form.

Glycerol Biosynthetic Pathway

It is contemplated that glycerol may be produced in recombinant organisms by the manipulation of the glycerol biosynthetic pathway found in most microorganisms. Typically, a carbon substrate such as glucose is converted to glucose-6-phosphate via hexokinase in the presence of ATP. Glucose-phosphate isomerase catalyzes the conversion of glucose-6-phosphate to fructose-6-phosphate and then to fructose-1,6-diphosphate through the action of 6-phosphofructokinase. The diphosphate is then taken to dihydroxyacetone phosphate (DHAP) via aldolase. Finally NADH-dependent G3PDH converts DHAP to glycerol-3-phosphate which is then dephosphorylated to glycerol by G3P phosphatase. (Agarwal (1990), *Adv. Biochem. Engrg.* 41:114).

Genes encoding G3PDH, glycerol dehydrogenase, G3P phosphatase and glycerol kinase

The present invention provides genes suitable for the expression of G3PDH and G3P phosphatase activities in a host cell.

Genes encoding G3PDH are known. For example, GPD1 has been isolated from *Saccharomyces* and has the base sequence given by SEQ ID NO:1, encoding the amino acid sequence given in SEQ ID NO:7 (Wang et al., *supra*). Similarly, G3PDH activity has also been isolated from *Saccharomyces* encoded by GPD2 having the base sequence given in SEQ ID NO:2 encoding the amino acid sequence given in SEQ ID NO:8 (Eriksson et al., (1995) *Mol. Microbiol.*, 17:95).

For the purposes of the present invention it is contemplated that any gene encoding a polypeptide responsible for G3PDH activity is suitable wherein that activity is capable of catalyzing the conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P). Further, it is contemplated that any gene encoding the amino acid sequence of G3PDH as given by SEQ ID NOS:7, 8, 9, 10, 11 and 12 corresponding to the genes GPD1, GPD2, GUT2, gpaA, glpD, and the α subunit of glpABC respectively, will be functional in the present invention wherein that amino acid sequence may encompass amino acid substitutions, deletions or additions that do not alter the function of the enzyme. The skilled person will appreciate that genes encoding G3PDH isolated from other sources will also be suitable for use in the present invention. For

example, genes isolated from prokaryotes include GenBank accessions M34393, M20938, L01111, U12567, L45246, L45323, L45371, L45325, U32164, U32689, and U39682. Genes isolated from fungi include GenBank accessions U30625, U30876 and X56162; genes isolated from insects include GenBank
5 accessions X61223 and X14179; and genes isolated from mammalian sources include GenBank accessions U12424, M25558 and X78593.

Genes encoding G3P phosphatase are known. For example, GPP2 has been isolated from *Saccharomyces cerevisiae* and has the base sequence given by SEQ ID NO:5, which encodes the amino acid sequence given in SEQ ID NO:13
10 (Norbeck et al., (1996), *J. Biol. Chem.*, 271:13875).

For the purposes of the present invention, any gene encoding a G3P phosphatase activity is suitable for use in the method wherein that activity is capable of catalyzing the conversion of glycerol-3-phosphate and water to glycerol and inorganic phosphate. Further, any gene encoding the amino acid
15 sequence of G3P phosphatase as given by SEQ ID NOS:13 and 14 corresponding to the genes GPP2 and GPP1 respectively, will be functional in the present invention including any amino acid sequence that encompasses amino acid substitutions, deletions or additions that do not alter the function of the G3P
20 phosphatase enzyme. The skilled person will appreciate that genes encoding G3P phosphatase isolated from other sources will also be suitable for use in the present invention. For example, the dephosphorylation of glycerol-3-phosphate to yield glycerol may be achieved with one or more of the following general or
specific phosphatases: alkaline phosphatase (EC 3.1.3.1) [GenBank M19159, M29663, U02550 or M33965]; acid phosphatase (EC 3.1.3.2) [GenBank
25 U51210, U19789, U28658 or L20566]; glycerol-3-phosphatase (EC 3.1.3.-) [GenBank Z38060 or U18813x11]; glucose-1-phosphatase (EC 3.1.3.10) [GenBank M33807]; glucose-6-phosphatase (EC 3.1.3.9) [GenBank U00445];
fructose-1,6-bisphosphatase (EC 3.1.3.11) [GenBank X12545 or J03207] or phosphatidyl glycerol phosphate phosphatase (EC 3.1.3.27) [GenBank M23546
30 and M23628].

Genes encoding glycerol kinase are known. For example, GUT1 encoding the glycerol kinase from *Saccharomyces* has been isolated and sequenced (Pavlik et al. (1993), *Curr. Genet.*, 24:21) and the base sequence is
35 given by SEQ ID NO:6, which encodes the amino acid sequence given in SEQ ID NO:15. Alternatively, *glpK* encodes a glycerol kinase from *E. coli* and is characterized by the base sequence given in GeneBank L19201, base pairs 77347-78855.

Genes encoding glycerol dehydrogenase are known. For example, *gldA* encodes a glycerol dehydrogenase from *E. coli* and is characterized by the base

sequence given in GeneBank U00006, base pairs 3174-4316. Alternatively, *dhaD* refers to another gene that encodes a glycerol dehydrogenase from *Citrobacter freundii* and is characterized by the base sequence given in GeneBank U09771, base pairs 2557-3654.

5 Host cells

Suitable host cells for the recombinant production of glycerol by the expression of G3PDH and G3P phosphatase may be either prokaryotic or eukaryotic and will be limited only by their ability to express active enzymes. Preferred host cells will be those bacteria, yeasts, and filamentous fungi typically useful for the production of glycerol such as *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*. Preferred in the present invention are *E. coli* and *Saccharomyces*.

Where glycerol is a key intermediate in the production of 1,3-propanediol the host cell will either have an endogenous gene encoding a protein having a dehydratase activity or will acquire such a gene through transformation. Host cells particularly suited for production of 1,3-propanediol are *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, and *Salmonella*, which have endogenous genes encoding dehydratase enzymes. Additionally, host cells that lack such an endogeneous gene include *E. coli*.

20 Vectors And Expression Cassettes

The present invention provides a variety of vectors and transformation and expression cassettes suitable for the cloning, transformation and expression of G3PDH and G3P phosphatase into a suitable host cell. Suitable vectors will be those which are compatible with the bacterium employed. Suitable vectors can be derived, for example, from a bacteria, a virus (such as bacteriophage T7 or a M-13 derived phage), a cosmid, a yeast or a plant. Protocols for obtaining and using such vectors are known to those in the art (Sambrook et al., Molecular Cloning: A Laboratory Manual - volumes 1, 2, 3 (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989)).

Typically, the vector or cassette contains sequences directing transcription and translation of the appropriate gene, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. It is most preferred when both control regions are derived from genes homologous to the transformed host cell. Such control

regions need not be derived from the genes native to the specific species chosen as a production host.

Initiation control regions, or promoters, which are useful to drive expression of the G3PDH and G3P phosphatase genes in the desired host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving these genes is suitable for the present invention including but not limited to CYC1, HIS3, GAL1, GAL10, ADH1, PGK, PHO5, GAPDH, ADC1, TRP1, URA3, LEU2, ENO, and TPI (useful for expression in *Saccharomyces*); AOX1 (useful for expression in *Pichia*); and lac, trp, λP_L , λP_R , T7, tac, and trc, (useful for expression in *E. coli*).

Termination control regions may also be derived from various genes native to the preferred hosts. Optionally, a termination site may be unnecessary; however, it is most preferred if included.

For effective expression of the instant enzymes, DNA encoding the enzymes are linked operably through initiation codons to selected expression control regions such that expression results in the formation of the appropriate messenger RNA.

Transformation Of Suitable Hosts And Expression Of G3PDH And G3P Phosphatase For The Production Of Glycerol

Once suitable cassettes are constructed they are used to transform appropriate host cells. Introduction of the cassette containing the genes encoding G3PDH and/or G3P phosphatase into the host cell may be accomplished by known procedures such as by transformation, e.g., using calcium-permeabilized cells, electroporation, or by transfection using a recombinant phage virus (Sambrook et al., *supra*).

In the present invention AH21 and DAR1 cassettes were used to transform the *E. coli* DH5 α and FM5 as fully described in the GENERAL METHODS and EXAMPLES.

Random And Site Specific Mutagenesis For Disrupting Enzyme Activities:

Enzyme pathways by which organisms metabolize glycerol are known in the art, Figure 1. Glycerol is converted to glycerol-3-phosphate (G3P) by an ATP-dependent glycerol kinase; the G3P may then be oxidized to DHAP by G3PDH. In a second pathway, glycerol is oxidized to dihydroxyacetone (DHA) by a glycerol dehydrogenase; the DHA may then be converted to DHAP by an ATP-dependent DHA kinase. In a third pathway, glycerol is oxidized to glyceraldehyde by a glycerol dehydrogenase; the glyceraldehyde may be phosphorylated to glyceraldehyde-3-phosphate by an ATP-dependent kinase. DHAP and glyceraldehyde-3-phosphate, interconverted by the action of triosephosphate isomerase, may be further metabolized via central metabolism

pathways. These pathways, by introducing by-products, are deleterious to glycerol production.

One aspect of the present invention is the ability to provide a production organism for the production of glycerol where the glycerol-converting activities of glycerol kinase and glycerol dehydrogenase have been deleted. Methods of creating deletion mutants are common and well known in the art. For example, wild type cells may be exposed to a variety of agents such as radiation or chemical mutagens and then screened for the desired phenotype. When creating mutations through radiation either ultraviolet (UV) or ionizing radiation may be used. Suitable short wave UV wavelengths for genetic mutations will fall within the range of 200 nm to 300 nm where 254 nm is preferred. UV radiation in this wavelength principally causes changes within nucleic acid sequence from guanine and cytosine to adenine and thymidine. Since all cells have DNA repair mechanisms that would repair most UV induced mutations, agents such as caffeine and other inhibitors may be added to interrupt the repair process and maximize the number of effective mutations. Long wave UV mutations using light in the 300 nm to 400 nm range are also possible but are generally not as effective as the short wave UV light unless used in conjunction with various activators such as psoralen dyes that interact with the DNA.

Mutagenesis with chemical agents is also effective for generating mutants and commonly used substances include chemicals that affect nonreplicating DNA such as HNO_2 and NH_2OH , as well as agents that affect replicating DNA such as acridine dyes, notable for causing frameshift mutations. Specific methods for creating mutants using radiation or chemical agents are well documented in the art. See for example Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA., or Deshpande, Mukund V., *Appl. Biochem. Biotechnol.*, 36, 227, (1992), herein incorporated by reference.

After mutagenesis has occurred, mutants having the desired phenotype may be selected by a variety of methods. Random screening is most common where the mutagenized cells are selected for the ability to produce the desired product or intermediate. Alternatively, selective isolation of mutants can be performed by growing a mutagenized population on selective media where only resistant colonies can develop. Methods of mutant selection are highly developed and well known in the art of industrial microbiology. See Brock, *Supra.*, DeMancilha et al., *Food Chem.*, 14, 313, (1984).

Biological mutagenic agents which target genes randomly are well known in the art. See for example De Bruijn and Rossbach in Methods for General and Molecular Bacteriology (1994) American Society for Microbiology,

Washington, D.C. Alternatively, provided that gene sequence is known, chromosomal gene disruption with specific deletion or replacement is achieved by homologous recombination with an appropriate plasmid. See for example Hamilton et al. (1989) *J. Bacteriol.* 171:4617-4622, Balbas et al. (1993) *Gene* 136: 211-213, Gueldener et al. (1996) *Nucleic Acids Res.* 24: 2519-2524, and Smith et al. (1996) *Methods Mol. Cell. Biol.* 5: 270-277.

It is contemplated that any of the above cited methods may be used for the deletion or inactivation of glycerol kinase and glycerol dehydrogenase activities in the preferred production organism.

10 Media and Carbon Substrates

Fermentation media in the present invention must contain suitable carbon substrates. Suitable substrates may include but are not limited to mono-saccharides such as glucose and fructose, oligosaccharides such as lactose or sucrose, polysaccharides such as starch or cellulose or mixtures thereof and unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. Additionally, the carbon substrate may also be one-carbon substrates such as carbon dioxide, or methanol for which metabolic conversion into key biochemical intermediates has been demonstrated.

20 Glycerol production from single carbon sources (e.g., methanol, formaldehyde or formate) has been reported in methylotrophic yeasts (Yamada et al. (1989), *Agric. Biol. Chem.*, 53(2):541-543) and in bacteria (Hunter et al. (1985), *Biochemistry*, 24:4148-4155). These organisms can assimilate single carbon compounds, ranging in oxidation state from methane to formate, and produce glycerol. The pathway of carbon assimilation can be through ribulose monophosphate, through serine, or through xylulose-monophosphate (Gottschalk, Bacterial Metabolism, Second Edition, Springer-Verlag: New York (1986)). The ribulose monophosphate pathway involves the condensation of formate with ribulose-5-phosphate to form a 6 carbon sugar that becomes fructose and eventually the three carbon product, glyceraldehyde-3-phosphate. Likewise, the serine pathway assimilates the one-carbon compound into the glycolytic pathway via methylenetetrahydrofolate.

35 In addition to one and two carbon substrates, methylotrophic organisms are also known to utilize a number of other carbon-containing compounds such as methylamine, glucosamine and a variety of amino acids for metabolic activity. For example, methylotrophic yeast are known to utilize the carbon from methylamine to form trehalose or glycerol (Bellion et al. (1993), *Microb. Growth C1 Compd.*, [Int. Symp.], 7th, 415-32. Editor(s): Murrell, J. Collin; Kelly, Don P. Publisher: Intercept, Andover, UK). Similarly, various species

of *Candida* will metabolize alanine or oleic acid (Sulter et al. (1990), *Arch. Microbiol.*, 153(5):485-9). Hence, the source of carbon utilized in the present invention may encompass a wide variety of carbon-containing substrates and will only be limited by the choice of organism.

5 Although all of the above mentioned carbon substrates and mixtures thereof are suitable in the present invention, preferred carbon substrates are monosaccharides, oligosaccharides, polysaccharides, single-carbon substrates or mixtures thereof. More preferred are sugars such as glucose, fructose, sucrose, maltose, lactose and single carbon substrates such as methanol and carbon
10 dioxide. Most preferred as a carbon substrate is glucose.

 In addition to an appropriate carbon source, fermentation media must contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for the growth of the cultures and promotion of the enzymatic pathway necessary for glycerol production.

15 Culture Conditions

 Typically cells are grown at 30 °C in appropriate media. Preferred growth media are common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth, or Yeast medium (YM) broth. Other defined or synthetic growth media may also be used and the appropriate
20 medium for growth of the particular microorganism will be known by one skilled in the art of microbiology or fermentation science. The use of agents known to modulate catabolite repression directly or indirectly, e.g., cyclic adenosine 3':5'-monophosphate, may also be incorporated into the reaction media. Similarly, the use of agents known to modulate enzymatic activities
25 (e.g., sulfites, bisulfites, and alkalis) that lead to enhancement of glycerol production may be used in conjunction with or as an alternative to genetic manipulations.

 Suitable pH ranges for the fermentation are between pH 5.0 to pH 9.0 where the range of pH 6.0 to pH 8.0 is preferred for the initial condition.

30 Reactions may be performed under aerobic or anaerobic conditions where anaerobic or microaerobic conditions are preferred.

Identification of G3PDH, glycerol dehydrogenase, G3P phosphatase, and glycerol kinase activities

 The levels of expression of the proteins G3PDH, G3P phosphatase
35 glycerol dehydrogenase, and glycerol kinase are measured by enzyme assays. Generally, G3PDH activity and glycerol dehydrogenase activity assays rely on the spectral properties of the cosubstrate, NADH, in the DHAP conversion to G-3-P and the DHA conversion to glycerol, respectively. NADH has intrinsic UV/vis absorption and its consumption can be monitored spectrophotometrically

at 340 nm. G3P phosphatase activity can be measured by any method of measuring the inorganic phosphate liberated in the reaction. The most commonly used detection method uses the visible spectroscopic determination of a blue-colored phosphomolybdate ammonium complex. Glycerol kinase activity can be measured by the detection of G3P from glycerol and ATP, for example, by NMR. Assays can be directed toward more specific characteristics of individual enzymes if necessary, for example, by the use of alternate cofactors.

Identification and recovery of glycerol and other products (e.g. 1,3-propanediol)

Glycerol and other products (e.g. 1,3-propanediol) may be identified and quantified by high performance liquid chromatography (HPLC) and gas chromatography/mass spectroscopy (GC/MS) analyses on the cell-free extracts. Preferred is a HPLC method where the fermentation media are analyzed on an analytical ion exchange column using a mobile phase of 0.01N sulfuric acid in an isocratic fashion.

Methods for the recovery of glycerol from fermentation media are known in the art. For example, glycerol can be obtained from cell media by subjecting the reaction mixture to the following sequence of steps: filtration; water removal; organic solvent extraction; and fractional distillation (U.S. Patent No. 2,986,495).

Description Of The Preferred Embodiments

Production of Glycerol

The present invention describes a method for the production of glycerol from a suitable carbon source utilizing a recombinant organism. Particularly suitable in the invention is a bacterial host cell, transformed with an expression cassette carrying either or both of a gene that encodes a protein having a glycerol-3-phosphate dehydrogenase activity and a gene encoding a protein having a glycerol-3-phosphatase activity. In addition to the G3PDH and G3P phosphatase genes, the host cell will contain disruptions in either or both of genes encoding endogenous glycerol kinase and glycerol dehydrogenase enzymes. The combined effect of the foreign G3PDH and G3P phosphatase genes (providing a pathway from the carbon source to glycerol) with the gene disruptions (blocking the conversion of glycerol) results in an organism that is capable of efficient and reliable glycerol production.

Although the optimal organism for glycerol production contains the above mentioned gene disruptions, glycerol production is possible with a host cell containing either one or both of the foreign G3PDH and G3P phosphatase genes in the absence of such disruptions. For example, the recombinant *E. coli* strain AA200 carrying the DAR1 gene (Example 1) was capable of producing between 0.38 g/L and 0.48 g/L of glycerol depending on fermentation

parameters. Similarly, the *E. coli* DH5 α , carrying and expressible GPP2 gene (Example 2), was capable of 0.2 g/L of glycerol production. Where both genes are present, (Example 3 and 4), glycerol production attained about 40 g/L. Where both genes are present in conjunction with an elimination of the endogenous glycerol kinase activity, a reduction in the conversion of glycerol may be seen (Example 8). Furthermore, the presence of glycerol dehydrogenase activity is linked to the conversion of glycerol under glucose-limited conditions; thus, it is anticipated that the elimination of glycerol dehydrogenase activity will result in the reduction of glycerol conversion (Example 8).

Production of 1,3-propanediol

The present invention may also be adapted for the production of 1,3-propanediol by utilizing recombinant organisms expressing the foreign G3PDH and/or G3P phosphatase genes and containing disruptions in the endogenous glycerol kinase and/or glycerol dehydrogenase activities.

Additionally, the invention provides for the process for the production of 1,3-propanediol from a recombinant organism where multiple copies of endogeneous genes are introduced. In addition to these genetic alterations, the production cell will require the presence of a gene encoding an active dehydratase enzyme. The dehydratase enzyme activity may either be a glycerol dehydratase or a diol dehydratase. The dehydratase enzyme activity may result from either the expression of an endogenous gene or from the expression of a foreign gene transfected into the host organism. Isolation and expression of genes encoding suitable dehydratase enzymes are well known in the art and are taught by applicants in PCT/US96/06705, filed 5 November 1996 and U.S. 5686276 and U.S. 5633362, hereby incorporated by reference. It will be appreciated that, as glycerol is a key intermediate in the production of 1,3-propanediol, where the host cell contains a dehydratase activity in conjunction with expressed foreign G3PDH and/or G3P phosphatase genes and in the absence of the glycerol-converting glycerol kinase or glycerol dehydrogenase activities, the cell will be particularly suited for the production of 1,3-propanediol.

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLES

GENERAL METHODS

Procedures for phosphorylations, ligations, and transformations are well known in the art. Techniques suitable for use in the following examples may be found in Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989).

Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the following examples may be found in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994) or in Biotechnology: A Textbook of Industrial Microbiology (Thomas D. Brock, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA). All reagents and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

The meaning of abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), "mL" means milliliters, "L" means liters.

Cell strains

The following *Escherichia coli* strains were used for transformation and expression of G3PDH and G3P phosphatase. Strains were obtained from the *E. coli* Genetic Stock Center, ATCC, or Life Technologies (Gaithersburg, MD).

AA200 (*garB10 fhuA22 ompF627 fadL701 relA1 pit-10 spoT1 tpi-1 phoM510 mcrB1*) (Anderson et al., (1970), *J. Gen. Microbiol.*, 62:329).

BB20 (*tonA22 ΔphoA8 fadL701 relA1 glpR2 glpD3 pit-10 gpsA20 spoT1 T2R*) (Cronan et al., *J. Bact.*, 118:598).

DH5α (*deoR endA1 gyrA96 hsdR17 recA1 relA1 supE44 thi-1 Δ(lacZYA-argFV169) phi80lacZΔM15 F'*) (Woodcock et al., (1989), *Nucl. Acids Res.*, 17:3469).

FM5 *Escherichia coli* (ATCC 53911)

Identification of Glycerol

The conversion of glucose to glycerol was monitored by HPLC and/or GC. Analyses were performed using standard techniques and materials available to one of skill in the art of chromatography. One suitable method utilized a Waters Maxima 820 HPLC system using UV (210 nm) and RI detection. Samples were injected onto a Shodex SH-1011 column (8 mm x 300 mm; Waters, Milford, MA) equipped with a Shodex SH-1011P precolumn (6 mm x 50 mm), temperature-controlled at 50 °C, using 0.01 N H₂SO₄ as mobile phase at a flow rate of 0.69 mL/min. When quantitative analysis was desired, samples were prepared with a known amount of trimethylacetic acid as an external standard. Typically, the retention times of 1,3-propanediol (RI detection), glycerol (RI detection) and glucose (RI detection) were 21.39 min, 17.03 min and 12.66 min, respectively.

Glycerol was also analyzed by GC/MS. Gas chromatography with mass spectrometry detection for separation and quantitation of glycerol was performed using a DB-WAX column (30 m, 0.32 mm I.D., 0.25 um film thickness, J & W Scientific, Folsom, CA) at the following conditions: injector: split, 1:15; sample volume: 1 uL; temperature profile: 150 °C initial temperature with 30 sec hold, 40 °C/min to 180 °C, 20 °C/min to 240 °C, hold for 2.5 min. Detection: EI Mass Spectrometry (Hewlett Packard 5971, San Fernando, CA), quantitative SIM using ions 61 m/z and 64 m/z as target ions for glycerol and glycerol-d8, and ion 43 m/z as qualifier ion for glycerol. Glycerol-d8 was used as an internal standard.

Assay for glycerol-3-phosphatase, G3P phosphatase

The assay for enzyme activity was performed by incubating the extract with an organic phosphate substrate in a bis-Tris or MES and magnesium buffer, pH 6.5. The substrate used was either l- α -glycerol phosphate, or d,l- α -glycerol phosphate. The final concentrations of the reagents in the assay are: buffer (20 mM bis-Tris or 50 mM MES); MgCl₂ (10 mM); and substrate (20 mM). If the total protein in the sample was low and no visible precipitation occurs with an acid quench, the sample was conveniently assayed in the cuvette. This method involved incubating an enzyme sample in a cuvette that contained 20 mM substrate (50 μ L, 200 mM), 50 mM MES, 10 mM MgCl₂, pH 6.5 buffer. The final phosphatase assay volume was 0.5 mL. The enzyme-containing sample was added to the reaction mixture; the contents of the cuvette were mixed and then the cuvette was placed in a circulating water bath at T = 37 °C for 5 to 120 min, the length of time depending on whether the phosphatase activity in the enzyme sample ranged from 2 to 0.02 U/mL. The enzymatic reaction was quenched by the addition of the acid molybdate reagent

(0.4 mL). After the Fiske SubbaRow reagent (0.1 mL) and distilled water (1.5 mL) were added, the solution was mixed and allowed to develop. After 10 min, to allow full color development, the absorbance of the samples was read at 660 nm using a Cary 219 UV/Vis spectrophotometer. The amount of inorganic phosphate released was compared to a standard curve that was prepared by using a stock inorganic phosphate solution (0.65 mM) and preparing 6 standards with final inorganic phosphate concentrations ranging from 0.026 to 0.130 $\mu\text{mol/mL}$.

Spectrophotometric Assay for Glycerol 3-Phosphate Dehydrogenase (G3PDH)

10 Activity

The following procedure was used as modified below from a method published by Bell et al. (1975), *J. Biol. Chem.*, 250:7153-8. This method involved incubating an enzyme sample in a cuvette that contained 0.2 mM NADH; 2.0 mM dihydroxyacetone phosphate (DHAP), and enzyme in 0.1 M Tris/HCl, pH 7.5 buffer with 5 mM DTT, in a total volume of 1.0 mL at 30 °C. The spectrophotometer was set to monitor absorbance changes at the fixed wavelength of 340 nm. The instrument was blanked on a cuvette containing buffer only. After the enzyme was added to the cuvette, an absorbance reading was taken. The first substrate, NADH (50 μL 4 mM NADH; absorbance should increase approx 1.25 AU), was added to determine the background rate. The rate should be followed for at least 3 min. The second substrate, DHAP (50 μL 40 mM DHAP), was then added and the absorbance change over time was monitored for at least 3 min to determine the gross rate. G3PDH activity was defined by subtracting the background rate from the gross rate.

25 ^{13}C -NMR Assay for Glycerol Kinase Activity

An appropriate amount of enzyme, typically a cell-free crude extract, was added to a reaction mixture containing 40 mM ATP, 20 mM MgSO_4 , 21 mM uniformly ^{13}C labelled glycerol (99%, Cambridge Isotope Laboratories), and 0.1 M Tris-HCl, pH 9 for 75 min at 25 °C. The conversion of glycerol to glycerol 3-phosphate was detected by ^{13}C -NMR (125 MHz): glycerol (63.11 ppm, d, $J = 41$ Hz and 72.66 ppm, t, $J = 41$ Hz); glycerol 3-phosphate (62.93 ppm, d, $J = 41$ Hz; 65.31 ppm, br d, $J = 43$ Hz; and 72.66 ppm, dt, $J = 6, 41$ Hz).

NADH-linked Glycerol Dehydrogenase Assay

35 NADH -linked glycerol dehydrogenase activity in *E. coli* strains (*gldA*) was determined after protein separation by non-denaturing polyacrylamide gel electrophoresis. The conversion of glycerol plus NAD^+ to dihydroxyacetone plus NADH was coupled with the conversion of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to a deeply colored formazan, using

phenazine methosulfate (PMS) as mediator. (Yang et al. (1997) *J. Bacteriol.* 140:182).

Electrophoresis was performed in duplicate by standard procedures using native gels (8-16% TG, 1.5 mm, 15 lane gels from Novex, San Diego, CA).

- 5 Residual glycerol was removed from the gels by washing 3x with 50 mM Tris or potassium carbonate buffer, pH 9 for 10 min. The duplicate gels were developed, with and without glycerol (approx. 0.16 M final concentration), in 15 mL of assay solution containing 50 mM Tris or potassium carbonate, pH 9, 60 mg ammonium sulfate, 75 mg NAD⁺, 1.5 mg MTT, and 0.5 mg PMS.

- 10 The presence or absence of NADH-linked glycerol dehydrogenase activity in *E. coli* strains (*gldA*) was also determined, following polyacrylamide gel electrophoresis, by reaction with polyclonal antibodies raised to purified *K. pneumoniae* glycerol dehydrogenase (*dhaD*).

PLASMID CONSTRUCTION AND STRAIN CONSTRUCTION

- 15 Cloning and expression of glycerol 3-phosphatase for increase of glycerol production in *E. coli* DH5 α and FM5

- The *Saccharomyces cerevisiae* chromosome V lamda clone 6592 (Gene Bank, accession # U18813x11) was obtained from ATCC. The glycerol 3-phosphate phosphatase (GPP2) gene was cloned by cloning from the lamda clone as target DNA using synthetic primers (SEQ ID NO:16 with
20 SEQ ID NO:17) incorporating an BamHI-RBS-XbaI site at the 5' end and a SmaI site at the 3' end. The product was subcloned into pCR-Script (Stratagene, Madison, WI) at the SrfI site to generate the plasmids pAH15 containing GPP2. The plasmid pAH15 contains the GPP2 gene in the inactive orientation for
25 expression from the lac promoter in pCR-Script SK+. The BamHI-SmaI fragment from pAH15 containing the GPP2 gene was inserted into pBlueScriptII SK+ to generate plasmid pAH19. The pAH19 contains the GPP2 gene in the correct orientation for expression from the lac promoter. The XbaI-PstI fragment from pAH19 containing the GPP2 gene was inserted into pPHOX2 to
30 create plasmid pAH21. The pAH21/ DH5 α is the expression plasmid.

Plasmids for the over-expression of DAR1 in *E. coli*

- DAR1 was isolated by PCR cloning from genomic *S. cerevisiae* DNA using synthetic primers (SEQ ID NO:18 with SEQ ID NO:19). Successful PCR cloning places an NcoI site at the 5' end of DAR1 where the ATG within NcoI
35 is the DAR1 initiator methionine. At the 3' end of DAR1 a BamHI site is introduced following the translation terminator. The PCR fragments were digested with NcoI + BamHI and cloned into the same sites within the expression plasmid pTrc99A (Pharmacia, Piscataway, NJ) to give pDAR1A.

In order to create a better ribosome binding site at the 5' end of DAR1, an SpeI-RBS-NcoI linker obtained by annealing synthetic primers (SEQ ID NO:20 with SEQ ID NO:21) was inserted into the NcoI site of pDAR1A to create pAH40. Plasmid pAH40 contains the new RBS and DAR1 gene in the correct orientation for expression from the trc promoter of pTrc99A (Pharmacia, Piscataway, NJ). The NcoI-BamHI fragment from pDAR1A and an second set of SpeI-RBS-NcoI linker obtained by annealing synthetic primers (SEQ ID NO:22 with SEQ ID NO:23) was inserted into the SpeI-BamHI site of pBC-SK+ (Stratagene, Madison, WI) to create plasmid pAH42. The plasmid pAH42 contains a chloramphenicol resistant gene.

Construction of expression cassettes for DAR1 and GPP2

Expression cassettes for DAR1 and GPP2 were assembled from the individual DAR1 and GPP2 subclones described above using standard molecular biology methods. The BamHI-PstI fragment from pAH19 containing the ribosomal binding site (RBS) and GPP2 gene was inserted into pAH40 to create pAH43. The BamHI-PstI fragment from pAH19 containing the RBS and GPP2 gene was inserted into pAH42 to create pAH45.

The ribosome binding site at the 5' end of GPP2 was modified as follows. A BamHI-RBS-SpeI linker, obtained by annealing synthetic primers GATCCAGGAAACAGA (SEQ ID NO:24) with CTAGTCTGTTTCCTG (SEQ ID NO:25) to the XbaI-PstI fragment from pAH19 containing the GPP2 gene, was inserted into the BamHI-PstI site of pAH40 to create pAH48. Plasmid pAH48 contains the DAR1 gene, the modified RBS, and the GPP2 gene in the correct orientation for expression from the trc promoter of pTrc99A (Pharmacia, Piscataway, NJ).

Transformation of *E. coli*

All the plasmids described here were transformed into *E. coli* DH5 α or FM5 using standard molecular biology techniques. The transformants were verified by its DNA RFLP pattern.

EXAMPLE 1

PRODUCTION OF GLYCEROL FROM *E. COLI* TRANSFORMED WITH G3PDH GENE

Media

Synthetic media was used for anaerobic or aerobic production of glycerol using *E. coli* cells transformed with pDAR1A. The media contained per liter 6.0 g Na₂HPO₄, 3.0 g KH₂PO₄, 1.0 g NH₄Cl, 0.5 g NaCl, 1 mL 20% MgSO₄·7H₂O, 8.0 g glucose, 40 mg casamino acids, 0.5 ml 1% thiamine hydrochloride, 100 mg ampicillin.

Growth Conditions

Strain AA200 harboring pDAR1A or the pTrc99A vector was grown in aerobic conditions in 50 mL of media shaking at 250 rpm in 250 mL flasks at 37 °C. At A₆₀₀ 0.2-0.3 isopropylthio-β-D-galactoside was added to a final concentration of 1 mM and incubation continued for 48 h. For anaerobic growth samples of induced cells were used to fill Falcon #2054 tubes which were capped and gently mixed by rotation at 37 °C for 48 h. Glycerol production was determined by HPLC analysis of the culture supernatants. Strain pDAR1A/AA200 produced 0.38 g/L glycerol after 48 h under anaerobic conditions, and 0.48 g/L under aerobic conditions.

EXAMPLE 2

PRODUCTION OF GLYCEROL FROM *E. COLI* TRANSFORMED WITH G3P PHOSPHATASE GENE (GPP2)

Media

Synthetic phoA media was used in shake flasks to demonstrate the increase of glycerol by GPP2 expression in *E. coli*. The phoA medium contained per liter: Amisoy, 12 g; ammonium sulfate, 0.62 g; MOPS, 10.5 g; Na-citrate, 1.2 g; NaOH (1 M), 10 mL; 1 M MgSO₄, 12 mL; 100X trace elements, 12 mL; 50% glucose, 10 mL; 1% thiamine, 10 mL; 100 mg/mL L-proline, 10 mL; 2.5 mM FeCl₃, 5 mL; mixed phosphates buffer, 2 mL (5 mL 0.2 M NaH₂PO₄ + 9 mL 0.2 M K₂HPO₄), and pH to 7.0. The 100X traces elements for phoA medium /L contained: ZnSO₄·7 H₂O, 0.58 g; MnSO₄·H₂O, 0.34 g; CuSO₄·5 H₂O, 0.49 g; CoCl₂·6 H₂O, 0.47 g; H₃BO₃, 0.12 g, NaMoO₄·2 H₂O, 0.48 g.

Shake Flasks Experiments

The strains pAH21/DH5α (containing GPP2 gene) and pPHOX2/DH5α (control) were grown in 45 mL of media (phoA media, 50 ug/mL carbenicillin, and 1 ug/mL vitamin B₁₂) in a 250 mL shake flask at 37 °C. The cultures were grown under aerobic condition (250 rpm shaking) for 24 h. Glycerol production was determined by HPLC analysis of the culture supernatant. pAH21/DH5α produced 0.2 g/L glycerol after 24 h.

EXAMPLE 3

PRODUCTION OF GLYCEROL FROM D-GLUCOSE USING RECOMBINANT *E. COLI* CONTAINING BOTH GPP2 AND DAR1

Growth for demonstration of increased glycerol production by *E. coli* DH5α-containing pAH43 proceeds aerobically at 37 °C in shake-flask cultures (erlenmeyer flasks, liquid volume 1/5th of total volume).

Cultures in minimal media/1% glucose shake-flasks are started by inoculation from overnight LB/1% glucose culture with antibiotic selection.

Minimal media are: filter-sterilized defined media, final pH 6.8 (HCl), contained per liter: 12.6 g (NH₄)₂SO₄, 13.7 g K₂HPO₄, 0.2 g yeast extract (Difco), 1 g NaHCO₃, 5 mg vitamin B₁₂, 5 mL Modified Balch's Trace-Element Solution (the composition of which can be found in Methods for General and
 5 Molecular Bacteriology (P. Gerhardt et al., eds, p. 158, American Society for Microbiology, Washington, DC (1994)). The shake-flasks are incubated at 37 °C with vigorous shaking for overnight, after which they are sampled for GC analysis of the supernatant. The pAH43/DH5α showed glycerol production of 3.8 g/L after 24 h.

10 EXAMPLE 4

PRODUCTION OF GLYCEROL FROM D-GLUCOSE USING RECOMBINANT *E. COLI* CONTAINING BOTH GPP2 AND DAR1

Example 4 illustrates the production of glucose from the recombinant *E. coli* DH5α/pAH48, containing both the GPP2 and DAR1 genes.

15 The strain DH5α/pAH48 was constructed as described above in the GENERAL METHODS.

Pre-Culture

DH5α/pAH48 were pre-cultured for seeding into a fermentation run. Components and protocols for the pre-culture are listed below.

20 Pre-Culture Media

	KH ₂ PO ₄	30.0 g/L
	Citric acid	2.0 g/L
	MgSO ₄ ·7H ₂ O	2.0 g/L
	98% H ₂ SO ₄	2.0 mL/L
25	Ferric ammonium citrate	0.3 g/L
	CaCl ₂ ·2H ₂ O	0.2 g/L
	Yeast extract	5.0 g/L
	Trace metals	5.0 mL/L
	Glucose	10.0 g/L
30	Carbenicillin	100.0 mg/L

The above media components were mixed together and the pH adjusted to 6.8 with NH₄OH. The media was then filter sterilized.

Trace metals were used according to the following recipe:

	Citric acid, monohydrate	4.0 g/L
35	MgSO ₄ ·7H ₂ O	3.0 g/L
	MnSO ₄ ·H ₂ O	0.5 g/L
	NaCl	1.0 g/L
	FeSO ₄ ·7H ₂ O	0.1 g/L
	CoCl ₂ ·6H ₂ O	0.1 g/L

	CaCl ₂	0.1 g/L
	ZnSO ₄ ·7H ₂ O	0.1 g/L
	CuSO ₄ ·5 H ₂ O	10 mg/L
	AlK(SO ₄) ₂ ·12H ₂ O	10 mg/L
5	H ₃ BO ₃	10 mg/L
	Na ₂ MoO ₄ ·2H ₂ O	10 mg/L
	NiSO ₄ ·6H ₂ O	10 mg/L
	Na ₂ SeO ₃	10 mg/L
	Na ₂ WO ₄ ·2H ₂ O	10 mg/L

- 10 Cultures were started from seed culture inoculated from 50 µL frozen stock (15% glycerol as cryoprotectant) to 600 mL medium in a 2-L Erlenmeyer flask. Cultures were grown at 30 °C in a shaker at 250 rpm for approximately 12 h and then used to seed the fermenter.

Fermentation growth

15 Vessel

15-L stirred tank fermenter

Medium

	KH ₂ PO ₄	6.8 g/L
	Citric acid	2.0 g/L
20	MgSO ₄ ·7H ₂ O	2.0 g/L
	98% H ₂ SO ₄	2.0 mL/L
	Ferric ammonium citrate	0.3 g/L
	CaCl ₂ ·2H ₂ O	0.2 g/L
	Mazu DF204 antifoam	1.0 mL/L

- 25 The above components were sterilized together in the fermenter vessel. The pH was raised to 6.7 with NH₄OH. Yeast extract (5 g/L) and trace metals solution (5 mL/L) were added aseptically from filter sterilized stock solutions. Glucose was added from 60% feed to give final concentration of 10 g/L. Carbenicillin was added at 100 mg/L. Volume after inoculation was 6 L.

30 Environmental Conditions For Fermentation

- The temperature was controlled at 36 °C and the air flow rate was controlled at 6 standard liters per minute. Back pressure was controlled at 0.5 bar. The agitator was set at 350 rpm. Aqueous ammonia was used to control pH at 6.7. The glucose feed (60% glucose monohydrate) rate was controlled to maintain excess glucose.

Results

The results of the fermentation run are given in Table 1.

Table 1

EFT (hr)	OD550 (AU)	[Glucose] (g/L)	[Glycerol] (g/L)	Total Glucose Fed (g)	Total Glycerol Produced (g)
0	0.8	9.3		25	
6	4.7	4.0	2.0	49	14
8	5.4	0	3.6	71	25
10	6.7	0.0	4.7	116	33
12	7.4	2.1	7.0	157	49
14.2	10.4	0.3	10.0	230	70
16.2	18.1	9.7	15.5	259	106
18.2	12.4	14.5		305	
20.2	11.8	17.4	17.7	353	119
22.2	11.0	12.6		382	
24.2	10.8	6.5	26.6	404	178
26.2	10.9	6.8		442	
28.2	10.4	10.3	31.5	463	216
30.2	10.2	13.1	30.4	493	213
32.2	10.1	8.1	28.2	512	196
34.2	10.2	3.5	33.4	530	223
36.2	10.1	5.8		548	
38.2	9.8	5.1	36.1	512	233

EXAMPLE 5

ENGINEERING OF GLYCEROL KINASE MUTANTS OF *E. COLI* FM5
FOR PRODUCTION OF GLYCEROL FROM GLUCOSE

5 Construction of integration plasmid for glycerol kinase gene replacement in
E. coli FM5

E. coli FM5 genomic DNA was prepared using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN). A 1.0 kb DNA fragment containing partial *glpF* and glycerol kinase (*glpK*) genes was amplified by PCR

10 (Mullis and Faloona, *Methods Enzymol.*, 155:335-350, 1987) from FM5 genomic DNA using primers SEQ ID NO:26 and SEQ ID NO:27. A 1.1 kb DNA fragment containing partial *glpK* and *glpX* genes was amplified by PCR from FM5 genomic DNA using primers SEQ ID NO:28 and SEQ ID NO:29. A

15 *MunI* site was incorporated into primer SEQ ID NO:28. The 5' end of primer SEQ ID NO:28 was the reverse complement of primer SEQ ID NO:27 to enable subsequent overlap extension PCR. The gene splicing by overlap extension technique (Horton et al., *BioTechniques*, 8:528-535, 1990) was used to generate a 2.1 kb fragment by PCR using the above two PCR fragments as templates and

primers SEQ ID NO:26 and SEQ ID NO:29. This fragment represented a deletion of 0.8 kb from the central region of the 1.5 kb *glpK* gene. Overall, this fragment had 1.0 kb and 1.1 kb flanking regions on either side of the *MunI* cloning site (within the partial *glpK*) to allow for chromosomal gene replacement by homologous recombination.

The above 2.1 kb PCR fragment was blunt-ended (using mung bean nuclease) and cloned into the pCR-Blunt vector using the Zero Blunt PCR Cloning Kit (Invitrogen, San Diego, CA) to yield the 5.6 kb plasmid pRN100 containing kanamycin and Zeocin resistance genes. The 1.2 kb *HincII* fragment from pLoxCat1 (unpublished results), containing a chloramphenicol-resistance gene flanked by bacteriophage P1 *loxP* sites (Snaith et al., *Gene*, 166:173-174, 1995), was used to interrupt the *glpK* fragment in plasmid pRN100 by ligating it to *MunI*-digested (and blunt-ended) plasmid pRN100 to yield the 6.9 kb plasmid pRN101-1. A 376 bp fragment containing the R6K origin was amplified by PCR from the vector pGP704 (Miller and Mekalanos, *J. Bacteriol.*, 170:2575-2583, 1988) using primers SEQ ID NO:30 and SEQ ID NO:31, blunt-ended, and ligated to the 5.3 kb *Asp718-AatII* fragment (which was blunt-ended) from pRN101-1 to yield the 5.7 kb plasmid pRN102-1 containing kanamycin and chloramphenicol resistance genes. Substitution of the ColE1 origin region in pRN101-1 with the R6K origin to generate pRN102-1 also involved deletion of most of the Zeocin resistance gene. The host for pRN102-1 replication was *E. coli* SY327 (Miller and Mekalanos, *J. Bacteriol.*, 170:2575-2583, 1988) which contains the *pir* gene necessary for the function of the R6K origin.

25 Engineering Of Glycerol Kinase Mutant RJF10m With Chloramphenicol Resistance Gene Interrupt

E. coli FM5 was electrotransformed with the non-replicative integration plasmid pRN102-1 and transformants that were chloramphenicol-resistant (12.5 µg/mL) and kanamycin-sensitive (30 µg/mL) were further screened for glycerol non-utilization on M9 minimal medium containing 1 mM glycerol. An *EcoRI* digest of genomic DNA from one such mutant, RJF10m, when probed with the intact *glpK* gene via Southern analysis (Southern, *J. Mol. Biol.*, 98:503-517, 1975) indicated that it was a double-crossover integrant (*glpK* gene replacement) since the two expected 7.9 kb and 2.0 kb bands were observed, owing to the presence of an additional *EcoRI* site within the chloramphenicol resistance gene. The wild-type control yielded the single expected 9.4 kb band. A ¹³C NMR analysis of mutant RJF10m confirmed that it was incapable of converting ¹³C-labeled glycerol and ATP to glycerol-3-phosphate. This *glpK* mutant was further analyzed by genomic PCR using primer combinations SEQ

ID NO:32 and SEQ ID NO:33, SEQ ID NO:34 and SEQ ID NO:35, and SEQ ID NO:32 and SEQ ID NO:35 which yielded the expected 2.3 kb, 2.4 kb, and 4.0 kb PCR fragments respectively. The wild-type control yielded the expected 3.5 kb band with primers SEQ ID NO:32 and SEQ ID NO:35. The *glpK* mutant RJF10m was electrotransformed with plasmid pAH48 to allow glycerol production from glucose. The *glpK* mutant *E. coli* RJF10m has been deposited with ATCC under the terms of the Budapest Treaty on 24 November 1997.

Engineering Of Glycerol Kinase Mutant RJF10 With Chloramphenicol Resistance Gene Interrupt Removed

After overnight growth on YENB medium (0.75% yeast extract, 0.8% nutrient broth) at 37 °C, *E. coli* RJF10m in a water suspension was electrotransformed with plasmid pJW168 (unpublished results), which contained the bacteriophage P1 Cre recombinase gene under the control of the IPTG-inducible *lacUV5* promoter, a temperature-sensitive pSC101 replicon, and an ampicillin resistance gene. Upon outgrowth in SOC medium at 30 °C, transformants were selected at 30 °C (permissive temperature for pJW168 replication) on LB agar medium supplemented with carbenicillin (50 µg/mL) and IPTG (1 mM). Two serial overnight transfers of pooled colonies were carried out at 30 °C on fresh LB agar medium supplemented with carbenicillin and IPTG in order to allow excision of the chromosomal chloramphenicol resistance gene via recombination at the *loxP* sites mediated by the Cre recombinase (Hoess and Abremski, *J. Mol. Biol.*, 181:351-362, 1985). Resultant colonies were replica-plated on to LB agar medium supplemented with carbenicillin and IPTG and LB agar supplemented with chloramphenicol (12.5 µg/mL) to identify colonies that were carbenicillin-resistant and chloramphenicol-sensitive indicating marker gene removal. An overnight 30 °C culture of one such colony was used to inoculate 10 mL of LB medium. Upon growth at 30 °C to OD (600 nm) of 0.6, the culture was incubated at 37 °C overnight. Several dilutions were plated on prewarmed LB agar medium and the plates incubated overnight at 42 °C (the non-permissive temperature for pJW168 replication). Resultant colonies were replica-plated on to LB agar medium and LB agar medium supplemented with carbenicillin (75 µg/mL) to identify colonies that were carbenicillin-sensitive indicating loss of plasmid pJW168. One such *glpK* mutant, RJF10, was further analyzed by genomic PCR using primers SEQ ID NO:32 and SEQ ID NO:35 and yielded the expected 3.0 kb band confirming marker gene excision. Glycerol non-utilization by mutant RJF10 was confirmed by lack of growth on M9 minimal medium containing 1 mM glycerol. The *glpK* mutant RJF10 was electrotransformed with plasmid pAH48 to allow glycerol production from glucose.

EXAMPLE 6

CONSTRUCTION OF *E. COLI* STRAIN WITH *GLDA* GENE KNOCKOUT

The *gldA* gene was isolated from *E. coli* by PCR (K. B. Mullis and F. A. Faloona (1987) Meth. Enzymol. 155:335-350) using primers SEQ ID NO:36 and SEQ ID NO:37, which incorporate terminal Sph1 and Xba1 sites, respectively, and cloned (T. Maniatis 1982 Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, Cold Spring Harbor, NY) between the Sph1 and Xba1 sites in pUC18, to generate pKP8. pKP8 was cut at the unique Sal1 and Nco1 sites within the *gldA* gene, the ends flushed with Klenow and religated, resulting in a 109 bp deletion in the middle of *gldA* and regeneration of a unique Sal1 site, to generate pKP9. A 1.4 kb DNA fragment containing the gene conferring kanamycin resistance (kan), and including about 400 bps of DNA upstream of the translational start codon and about 100 bps of DNA downstream of the translational stop codon, was isolated from pET-28a(+) (Novagen, Madison, Wis) by PCR using primers SEQ ID NO:38 and SEQ ID NO:39, which incorporate terminal Sal1 sites, and subcloned into the unique Sal1 site of pKP9, to generate pKP13. A 2.1 kb DNA fragment beginning 204 bps downstream of the *gldA* translational start codon and ending 178 bps upstream of the *gldA* translational stop codon, and containing the kan insertion, was isolated from pKP13 by PCR using primers SEQ ID NO:40 and SEQ ID NO:41, which incorporate terminal Sph1 and Xba1 sites, respectively, was subcloned between the Sph1 and Xba1 sites in pMAK705 (Genencor International, Palo Alto, Calif.), to generate pMP33. *E. coli* FM5 was transformed with pMP33 and selected on 20 ug/mL kan at 30 °C, which is the permissive temperature for pMAK705 replication. One colony was expanded overnight at 30 °C in liquid media supplemented with 20 ug/mL kan. Approximately 32,000 cells were plated on 20 ug/mL kan and incubated for 16 hrs at 44 °C, which is the restrictive temperature for pMAK705 replication. Transformants growing at 44 °C have plasmid integrated into the chromosome, occurring at a frequency of approximately 0.0001. PCR and Southern blot (E.M. Southern 1975 *J. Mol. Biol.* 98:503-517) analyses were used to determine the nature of the chromosomal integration events in the transformants. Western blot analysis (H. Towbin, et al. (1979) *Proc. Natl. Acad. Sci.* 76:4350) was used to determine whether glycerol dehydrogenase protein, the product of *gldA*, is produced in the transformants. An activity assay was used to determine whether glycerol dehydrogenase activity remained in the transformants. Activity in glycerol dehydrogenase bands on native gels was determined by coupling the conversion of glycerol + NAD(+) → dihydroxyacetone + NADH to the conversion of a tetrazolium dye, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide] to a deeply colored formazan, with phenazine methosulfate as mediator. Glycerol dehydrogenase also requires the presence of 30 mM ammonium sulfate and 100 mM Tris, pH 9 (C.-T. Tang, et al. (1997) *J. Bacteriol.* 140:182). Of 8 transformants analyzed, 6 were determined to be *gldA* knockouts. *E. coli* MSP33.6 has been deposited with ATCC under the terms of the Budapest Treaty on 24 November 1997.

EXAMPLE 7

CONSTRUCTION OF *E. COLI* STRAIN WITH *GLPK* AND *GLDA* GENE KNOCKOUTS

10 A 1.6 kb DNA fragment containing the *gldA* gene, and including 228 bps of DNA upstream of the translational start codon and 220 bps of DNA downstream of the translational stop codon was isolated from *E. coli* by PCR using primers SEQ ID NO:42 and SEQ ID NO:43, which incorporate terminal Sph1 and Xba1 sites, respectively, and cloned between the Sph1 and Xba1 sites of pUC18, to generate pQN2. pQN2 was cut at the unique Sal1 and Nco1 sites within the *gldA* gene, the ends flushed with Klenow and religated, resulting in a 109 bp deletion in the middle of *gldA* and regeneration of a unique Sal1 site, to generate pQN4. A 1.2 kb DNA fragment containing the gene conferring kanamycin resistance (kan), and flanked by loxP sites was isolated from pLoxKan2 (Genecor International, Palo Alto, Calif.) as a Stu1/Xho1 fragment, the ends flushed with Klenow, and subcloned into pQN4 at the Sal1 site after flushing with Klenow, to generate pQN8. The Sph1/Xba1 fragment from pQN8 containing the kan-interrupted *gldA* was subcloned between the Sph1 and Xba1 sites of pGP704, using *E. coli* SY327 as host, to generate pQN9. *E. coli* RJF10 (see EXAMPLE 5) was transformed with pQN9 and selected on kan. Since the pGP704 backbone cannot replicate in most *E. coli* hosts, transformants arise by integration of the plasmid, (or portions of it) into the chromosome. Double crossover integration events are determined by identifying those transformants which are kan resistant and ampicillin sensitive. PCR and Southern blot analyses are used to determine the nature of the chromosomal integration events in the transformants. Western blot analysis is used to determine whether glycerol dehydrogenase, the product of *gldA*, is produced in the transformants. Activity assays are used to determine whether glycerol dehydrogenase activity remains in the transformants. The kan marker is removed from the chromosome using the Cre-producing plasmid pJW168, as described in EXAMPLE 5.

EXAMPLE 8
CONSUMPTION OF GLYCEROL PRODUCED FROM D-GLUCOSE BY
RECOMBINANT *E. COLI* CONTAINING BOTH GPP2 AND DAR1 WITH
AND WITHOUT GLYCEROL KINASE (*GLPK*) ACTIVITY

5 EXAMPLE 8 illustrates the consumption of glycerol by the recombinant *E. coli* FM5/pAH48 and RJF10/pAH48. The strains FM5/pAH48 and RJF10/pAH48 were constructed as described above in the GENERAL METHODS.

Pre-Culture

10 FM5/pAH48 and RJF10/pAH48 were pre-cultured for seeding a fermenter in the same medium used for fermentation, or in LB supplemented with 1% glucose. Either carbenicillin or ampicillin were used (100 mg/L) for plasmid maintenance. The medium for fermentation is as described in EXAMPLE 4.

15 Cultures were started from frozen stocks (15% glycerol as cryoprotectant) in 600 mL medium in a 2-L Erlenmeyer flask, grown at 30 °C in a shaker at 250 rpm for approximately 12 h, and used to seed the fermenter.

Fermentation growth

20 A 15-L stirred tank fermenter with 5-7 L initial volume was prepared as described in EXAMPLE 4. Either carbenicillin or ampicillin were used (100 mg/L) for plasmid maintenance.

Environmental Conditions to Evaluate Glycerol Kinase (*GlpK*) Activity

25 The temperature was controlled at 30 °C and the air flow rate controlled at 6 standard liters per minute. Back pressure was controlled at 0.5 bar. Dissolved oxygen tension was controlled at 10% by stirring. Aqueous ammonia was used to control pH at 6.7. The glucose feed (60% glucose) rate was controlled to maintain excess glucose until glycerol had accumulated to at least 25 g/L. Glucose was then depleted, resulting in the net metabolism of glycerol. Table 2 shows the resulting conversion of glycerol.

30

Table 2
Conversion of glycerol by FM5/pAH48 (wt) and RJF10/pAH48 (*glpK*)

Strain	number of examples	rate of glycerol consumption g/OD/hr
FM5/pAH48	2	0.095 ± 0.015
RJF10/pAH48	3	0.021 ± 0.011

As is seen by the data in Table 2, the rate of glycerol consumption decreases about 4-5 fold where endogenous glycerol kinase activity is eliminated.

Environmental Conditions to Evaluate Glycerol Dehydrogenase (GldA) Activity

5 The temperature was controlled at 30 °C and the air flow rate controlled at 6 standard liters per minute. Back pressure was controlled at 0.5 bar. Dissolved oxygen tension was controlled at 10% by stirring. Aqueous ammonia was used to control pH at 6.7. In the first fermentation, glucose was kept in excess for the duration of the fermentation. The second fermentation was
10 operated with no residual glucose after the first 25 hours. Samples over time from the two fermentations were taken for evaluation of GlpK and GldA activities. Table 3 summarizes RJF10/pAH48 fermentations that show the effects of GldA on selectivity for glycerol.

Table 3
GldA and GlpK activities from two RJF10/pAH48 fermentations

<u>Fermentation</u>	<u>Time (hrs)</u>	<u>GldA</u>	<u>GlpK</u>	<u>Overall selectivity (g/g)</u>
1	25	-	-	42%
	46	-	-	49%
	61	+	-	54%
2	25	+	-	41%
	46	++	-	14%
	61	++	-	12%

15 As is seen by the data in Table 3, the presence of glycerol dehydrogenase (GldA) activity is linked to the conversion of glycerol under glucose-limited conditions; thus, it is anticipated that eliminating glycerol dehydrogenase
20 activity will reduce glycerol conversion.